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Comparison of eNO and histamine hyperresponsiveness in diagnosing asthma in new referrals

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Summary
The mainstay of the diagnosis of asthma is the presence of reversible airway obstruction. Exhaled NO levels are increased in asthma, in close relationship with the amount of airway inflammation, and may be used for monitoring the disease and adjusting therapy. In this study we investigated the role of eNO as a diagnostic for asthma, compared with the FEV1-reversibility and the PC20 (20% decrease of the FEV1 in the bronchial histamine provocation test), in two independent centers, on an unselected population. ENO measurements were performed with chemoluminesence technique in one center and with an electrochemical device in the other. Only after correction for so-called nuisance factors (allergy, use of inhaled steroids, recent infection, smoking, sex and the use of nitrate food) the eNO appeared as a diagnostic with equal power as the FEV1-reversibility and the PC20.

Therefore, screening for asthma in our study population, with the eNO measurement, is a simple, fast and safe strategy.

Introduction

Asthma is a chronic inflammatory disorder, characterised by recurrent respiratory symptoms against a background of increased bronchial responsiveness to external stimuli, giving rise to variable airflow limitation which is reversible either spontaneously or with treatment.[1] Asthma is very common in Europe, its prevalence in young adults is estimated about 20%.[2] Diagnosis of asthma is based on symptoms and evidence of reversible airway obstruction, shown in pulmonary function tests. This may not always be straightforward and sometimes confirmation is needed by bronchial provocation tests (histamine or metacholine provocation tests). These tests are time-consuming and constitute a certain risk for patients as they can lead to severe bronchoconstriction.

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Exhaled NO (eNO) is increased in patients with asthma and can be used to monitor the therapeutic settings in asthma.[3,4] ENO also harbors the potential to serve as a diagnostic separating asthma from non-asthma.[5] The measurement of eNO is easy and safe to perform in both adults and children; most school-aged children are able to perform the test.[6] Airway eosinofilia, eNO and bronchial hyperresponsiveness are well correlated, as all are influenced by the same inflammatory process.[6,7] ENO levels are a strong predictor for steroid responsiveness in subjects with undiagnosed respiratory symptoms.[8] In a general population, eNO levels are strongly correlated with atopy.[9] Replacement of provocation tests by an eNO measurement would be advantageous, because the eNO test is quicker to perform and induces no bronchoconstriction.

The diagnostic role of eNO, however, is not well defined. Many researchers only correlated parameters, but did not test the equivalence of eNO to other diagnostic tests.[10,11] Especially comparisons of the histamine provocation test and eNO are scarce and suffer from spectrum or selection bias.[12,13] Therefore, data generated by these studies cannot simply be extrapolated to a population of possible asthmatics. In most studies with asthmatic subjects and eNO measurements, steroid naïve subjects were included. In daily practice general practitioners often start inhaled corticosteroid (ICS) therapy and subsequently refer to the pulmonologist. Therefore, the diagnostic quality of eNO in an unselected sample remains to be assessed.

This study examines the diagnostic role of eNO in comparison with the histamine provocation test and FEV1-reversibility, in two unselected samples of new referrals to a pulmonary outclinic from two different hospitals.

Methods

Centres

Two centres were involved in this study: the Spaarne Hospital, Hoofddorp, the Netherlands, which is a hospital serving the general community and the University Medical Center, Utrecht, the Netherlands (UMCU), which is a referral-type hospital, but also serves the general community.

Subjects

Random samples from all newly referred outpatients were drawn before a diagnosis was made. Immediately after a first visit to the outpatient clinic, subjects were invited to undergo full lung function testing, including measurement of the eNO levels and histamine provocation thresholds. Current medication was not a selection criterion, nor age or sex. Only those subjects referred for a suspected diagnosis in which measurement of eNO clearly is of no value were excluded (e.g. lung cancer). Subjects referred for a second opinion or in whom a diagnosis was already established were also excluded. Subjects gave written informed consent and the local medical ethical committees approved the study.

Pulmonary function measurements

Total lung capacity (TLC) and residual volume (RV) were determined by whole body plethysmography and spirometry/flow-volume curves via pneumotachography (Jaeger, Wurzburg and ZAN, Oberthulba, both in Germany) according to ERS-guidelines.[14] The V_A (alveolar volume), via single breath methane dilution, was measured as part of the determination of the TL_{CO} (Transfer factor of the lung for carbon monoxide).

At arrival, subjects first rested for $\pm$15 min after which the baseline lung function was determined. Three consecutive spirometry/flow-volume curves measurements were done and the flow-volume loop with the highest value of the FVC and FEV_1 was selected. Measurement of the bronchodilator response was done on a protocol basis.[14] On a second visit the eNO, allergy test and bronchoprovocation test was done consecutively. Patients refrained from using short-acting and long-acting bronchodilators 8 or 12 h prior to testing, respectively. All subjects received 400 μg salbutamol via MDI plus a spacer device, 15 min later spirometry was repeated, to measure/determine reversibility.

Measurement of exhaled NO

ENO measurements were performed in accordance with the ERS/ATS guidelines.[15] In the UMCU eNO levels were measured with a ECO MEDICS CLD 88 in conjunction with DENOX 88 (Eco Physics, Dürnten, Switserland). For measurements of eNO the subjects exhaled from total lung capacity to residual volume. The exhalation was controlled with a biofeedback monitor, and the subjects were asked to control the flow at 50 ml/s. Total exhalation time was 12 s. NO as well as CO_2 were measured. The point at which the CO_2 level reached 90% of its maximum was taken to determine the average NO over the next 5 s. Average of three measurements within 5% of each other were taken.

In the Spaarne Hospital eNO was measured with the Niox Mino device (Aerocrine, New Providence, United States of America), in which the NO is measured with an electrochemical cell, using an exhalation flow of 50 ml/s, with a total exhalation time of 10 s.

Measurement of histamine hyperresponsiveness

In the UMCU histamine diphosphate was administered using a DeVilbiss no. 646 handheld nebuliser (DeVilbiss Health Care Inc. Somerset, PA) in doubling doses from 0.25 to 16 mg/ml. Histamine and phosphate-buffered saline acted as positive and negative controls, respectively. The test was stopped at the moment the FEV_1 fell by more than 20%. Salbutamol aerosol was administered to aid recovery. The concentration of histamine that provoked a 20% fall in FEV1 (PC_{20}) was estimated by interpolation. Airway hyperresponsiveness was defined as a PC_{20} of $<$8 mg/ml.

In the Spaarne hospital the same protocol was used, with a Spira® Dosimeter (Spira Respiratory Care Center, Hämeenlinna, Finland), measurements were made on V_{max} Spectra 20 (Jaeger, Bilthoven, The Netherlands).

Skin prick testing

Sensitization to common allergens was measured by skin prick test reactions on the volar side of the forearm. In the UMCU fourteen allergens were tested (ALK-Abello AS Nieuwegein Netherlands). Mixed grasses 10.000 BU/ml, Mugwort (*Artemisia vulgaris)* 10.000 BU/ml, Mixed Tree pollen (birch, alder and hazel) 10.000 BU/ml, Dog (*Canis*

familiaris) 10.000 BU/ml, Cat (*Felis domesticus*) 10.000 BU/ml, Horse (*Equus caballus)* 10.000 BU/ml, *Aspergillus Fumigatus* 1:20 g/v, House dust mites (*Derm. Pteronyssinus* and *Derm. Farinae)* 10.000 BU/ml, Cavia (*Cavia porsellus)* 1:100 g/v, Rabbit (*Oryctolagus cuniculus)* 1:100 g/v, Parakeet (*Melopsittacus undulatus)* 1:20 g/v. Histamine and glycerol acted as positive and negative controls, respectively. In the Spaarne hospital only the first 8 allergens were used, also with positive and negative controls.

A positive reaction was defined as a 3 mm wheal or greater diameter 15 min after skin prick.

Parameters

The following items were scored via an interview:

1) use of inhaled steroids (drug, dose and type of inhaler)[16]
2) use of nitrate rich food <24 h (amount and time past since meal)[11]
3) respiratory infections <6 weeks (presence and duration)[15]
4) smoking (pack years)[17,18]

Diagnosis

The diagnosis, initially made by the treating physician, was reviewed by a panel of three independent pulmonologists. The diagnosis 'asthma' was based on integrative assessment of medical history, pulmonary function testing and bronchial hyperresponsiveness tests. Those not suffering from asthma were labelled as 'no asthma'. The eNO levels were not reported to the treating physician or to the panel, all other lung function parameters were available. Next to lung function testing, all other diagnostic measures were allowed and chosen at the discretion of the treating physician.

Statistics

We calculated means, standard deviations and 95% confidence intervals (CI) for normal distributed parameters and medians with 25%/75% quartiles for non-normal distributed ones. The eNO levels were ln-transformed to obtain a normal distributed parameter. Allergy, inhaled steroid use, use of nitrate rich food, respiratory infections and smoking were converted to either present (1) or absent (0).

The effects on eNO-, PC_{20} and FEV_1-reversibility of the above parameters were approached by unpaired *T*-tests. Logistic regression was subsequently used to calculate the probability to be an asthmatic: eNO-, PC_{20} or FEV_1-reversibility were the primary variables of interest. The presence of allergy, the use of inhaled steroids, the use of nitrate rich food, respiratory infections and smoking were considered as modifiers in the logistic regression analysis, as well as sex. Having obtained the probabilities, receiver operator curves (ROC) were constructed and the area under this curve estimated (which can be considered as the probability to make a correct diagnosis of the absence or presence of asthma). These areas were compared for statistical significant differences via the method of Metz using that of the PC_{20} as reference test.[19]

Table 1 List of non-asthmatic diagnosis.

	Spaarne	UMCU
Disease	%	%
Cough	25.0	19.3
COPD	6.8	18.5
Not obtained/Not specified	28.4	28.1
Sarcoidosis	4.5	3.7
Pleural disease	2.3	3.0
Pneumonia	10.2	3.0
Bronchiectasis	3.4	2.2
Hyperventilation	2.3	1.5
System disease	0	1.5
Pulmonary Embolism	2.3	0.7
Mesothelioma	0	0.7
Tumour	1.1	0.7
Vascular disease	0	0.7
Miscellaneous	13.6	16.4

Results

Subjects

In the Spaarne hospital 143 subjects were included of which 55 (38,5%) were labelled as 'asthmatic' by the panel. In the UMCU 219 subjects were included of which 85 (38,8%) were labelled as 'asthmatic'. The non-asthmatic diagnoses are depicted in Table 1.

In Table 2 the descriptive data of the subjects are displayed. The asthmatic group on both samples used inhaled steroids more frequently ($p < 0.001$), was more allergic ($p < 0.001$) and contained slightly less males in the UMCU ($p = 0.025$). However, the number of recent infections, or the use of nitrate food were all non-significantly different between the groups (data not shown). In the Spaarne Hospital the asthmatics were mostly non-smokers ($p = 0.004$), whereas in the UMCU no difference could be found.

Comparison of eNO, PC_{20} and FEV1-reversibility

Although the eNO levels were slightly higher in the Spaarne hospital compared to the UMCU, no significant parameter*hospital interaction or centre effect could be found for

Table 2 Descriptive data (mean, sd) of all subjects ($N = 362$) in the study (* = as percentage predicted).

	No asthma	Asthma	*p*-value
Age (years)	51.2 (12.7)	45.0 (11.0)	<0.001
Predilator FEV_1*	96.3 (18.9)	90.1 (16.1)	0.014
FEV_1/IVC	76.0 (9.6)	74.4 (10.2)	0.252
FEV_1-reversibility*	4.1 (5.2)	6.7 (6.1)	0.003
TLC*	105.0 (13.9)	103.8 (12.7)	0.525
RV*	111.6 (28.3)	112.3 (28.5)	0.876
Tl_{co}*	82.6 (19.0)	84.6 (16.0)	0.449

Table 3 Effect of various conditions on FEV_1-reversibility, eNO levels (ln-transformed) and PC_{20}.

Parameter		FEV1-reversibility			Exhaled NO (ln ppb)			PC_{20}	
		Mean	sd	*p*	Mean	sd	*p*	Median	p
Asthma	No	4.13	5.16	0.003	2.56	0.75	0.001	10.54	<0.001
	Yes	6.75	6.07		2.91	0.78		2.97	
Allergy	No	5.11	5.98	0.725	2.54	0.72	0.001	8.00	0.053
	Yes	5.42	5.12		2.88	0.81		5.65	
Use of inhaled steroids	No	5.11	5.49	0.863	2.79	0.76	0.010	6.60	0.829
	Yes	5.27	6.01		2.51	0.79		5.80	
Recent infection	No	5.39	6.00	0.358	2.64	0.80	0.073	6.90	0.096
	Yes	4.45	4.40		2.86	0.71		5.29	
Smoking	No	5.45	5.94	0.152	2.78	0.74	<0.001	7.55	0.027
	Yes	3.79	3.86		2.18	0.82		4.40	
Use of nitrate rich food	No	5.41	5.75	0.336	2.65	0.78	0.116	7.08	0.669
	Yes	4.43	5.42		2.84	0.75		5.67	
Sex	Female	4.57	5.87	0.190	2.52	0.72	<0.001	5.04	0.012
	Male	5.71	5.46		2.87	0.80		8.00	

eNO as well as the other parameters in the analysis: the differences between the yes/no groups hence do not differ between the two centres, therefore the data were pooled.

The ln-transformed eNO levels are statistically significant higher in asthma and allergy, lower in inhaled steroids users and smokers. The median PC_{20}-levels were statistically significant lower in asthma and smokers. The FEV_1-reversibility was higher only in asthmatics (see Table 3).

Diagnostic quality of exhaled NO, PC_{20} and FEV1-reversibility

Logistic regression was used to calculate the probability of being diagnosed as 'asthmatic', based on eNO, PC_{20} and FEV_1-reversibility. Allergy, use of inhaled steroids, recent infection, smoking, sex and the use of nitrate food were incorporated into the analysis as influencing factors. The resulting area under the ROC curve serves as main comparator (see Table 4) and the PC_{20} as the reference test.

As the findings from both centres were very similar we pooled the data to assess the equivalence between the methods under investigation. The differences between the PC_{20} area on one hand and the corrected eNO levels or FEV_1-reversibility on the other were non-significant (see Table 5). When we compared eNO without a correction for the modifiers, it turned to be significantly lower than the PC_{20} ROC area (Fig. 1).

Discussion

Summary

This study showed that eNO, FEV_1-reversibility and the PC_{20} can equally well discriminate asthma from non-asthma in unselected and frequently steroid-using population of new referred subjects to a pulmonary outclinic, only when eNO levels are corrected for the influence of its modifiers. This finding is present in an academic teaching hospital as well as in a general hospital. Without correcting for the nuisance factors, eNO is not a very usable tool in the diagnosis of asthma in this population.

Explanation

As said in the introduction, several eNO level modifiers are known and this study clearly confirms their effects in a group of unselected new referrals. Based on these findings and the underlying literature, we consider the effects of these modifiers as consistent and predictable and so the possibility emerges to correct for such modifiers.

Standard statistical methods, like logistic regression, offer validated approaches to correct for the influence of such modifiers: the effects of, e.g., medication and allergy can be accounted for. This will only expand the usability of eNO and makes exclusion of inhaled steroid users unnecessary. The alternative, to wash-out inhaled steroids and

Table 4 Area under the ROC for PC_{20}, eNO, uncorrected eNO and FEV_1-reversibility (as percent predicted). The AUC ROC denotes the probability to make a correct diagnosis of the absence or presence of asthma (scaled between minimal 0.5 and maximal 1); the *p*-value denotes the outcome of the comparison versus an AUC of 0.5, which indicates a useless test.

	Spaarne		UMCU	
	AUC ROC	*p*-value	AUC ROC	*p*-value
PC_{20}	0.874	<0.001	0.832	<0.001
eNO (corrected)	0.826	<0.001	0.821	<0.001
FEV_1-reversibility	0.805	<0.001	0.828	<0.001
eNO (uncorrected)	0.635	<0.001	0.641	0.002

Table 5 Comparison of AUC ROC curve of eNO, uncorrected eNO and FEV_1-reversibility (as percent predicted) using the PC_{20} AUC ROC as reference test (pooled data). The 95% CI is the confidence interval of the differences between the PC_{20} AUC ROC on one hand and the other parameters AUC ROC on the other. See Table 4 for AUC ROC values.

	p-value	95% confidence interval	
		lower bound	Upper bound
eNO	0.665	−0.043	0.067
eNO (uncorrected)	<0.001	0.110	0.320
FEV_1-reversibility	0.654	−0.041	0.065

apply the eNO test later in time, is very patient unfriendly, because it requires patients to refrain weeks from inhaled steroids. Similar goes for smoking, nitrate rich food and infections, while the presence of allergy cannot be avoided. When such requirements are necessary, the eNO test becomes hard to use in daily practice.

Logistic regression model

The relationship between eNO and inhaled steroids is a dose—response relationship, but we here used the straightforward presence or absence of ICS use as nuisance parameter. The drawback of modelling dose—response curves is the necessity for very large sample sizes to obtain sufficient power to cope with the range of dosages, the types of drugs and inhalers. A rule of thumb requires for each variable inserted into the analysis at least 20 cases. This disadvantage could be solved by converting drugs, doses and inhalers to a standard drug/dose/inhaler (e.g. beclomethasone dose equivalents), but that is a hazardous approach.

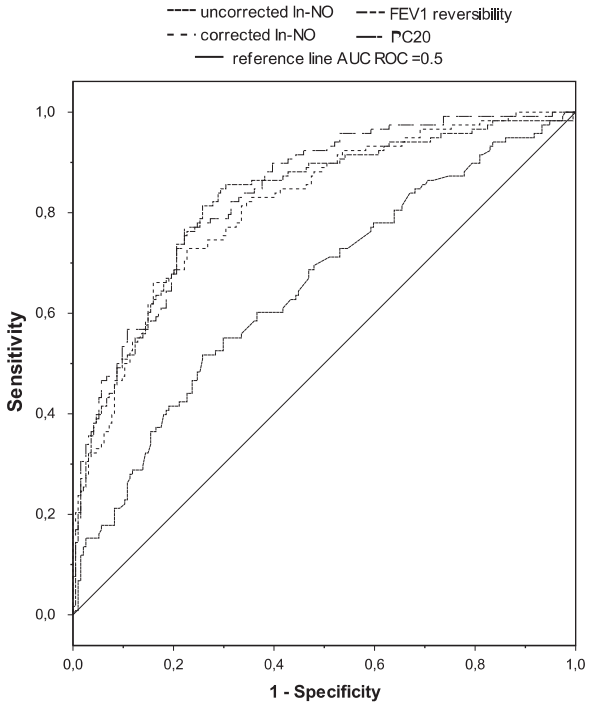

Figure 1 ROC curves of PC20, eNO, uncorrected eNO and FEV1-reversibility (as percent predicted).

The approach taken here was to categorize ICS use into 'yes/no', an approach often used in epidemiology and in eNO research.[20] The implicit assumption is that ICS lower eNO levels to a maximum degree irrespective of drug-potency, -dose and type of inhaler. This might well be the case when the reduction of eNO by steroids is an all-or-nothing phenomenon: when a low dose of ICS (lower than used in daily routine) already lowers eNO by a significant amount and higher doses not any further (=dose—response curve with an early plateau-phase), this requirement is fulfilled and the approach is valid. We feel that this is the case as several studies did show this phenomenon. In a randomized study in asthmatics Dal Negro could not show differences in eNO-response to 100 μg or 400 μg bid beclomethasone.[16] Kharitonov compared 100 μg and 400 μg budesonide and found differences between the dose-groups after 2 weeks of ICS: mean reduction 6.5 ppb versus 5.3 ppb after 400 μg and 100 μg budesonide.[21] In a study by Silkoff the conclusion was that in a low baseline eNO-group (60—100 ppb) 100 μg beclomethasone elicited a modest change, but that higher doses did not lower the eNO any further.[22] Only in the high eNO-group (>100 ppb) a dose—response relation with doses >100 μg was visible. Jatakanon investigated the effect of increasing budesonide doses on eNO and reported on 100 μg a −0.2 fold change, while on 400 μg and 1600 μg a −0.6 and respectively a −0.5 fold change occurred.[23]

Validation

The diagnosis of asthma, of course, plays an important role in this study. Despite the fact that a panel reviewed the diagnosis, it is realized that mistakes are still possible. This causes an imperfect standard bias and this error lowers the sensitivity and specificity of all test methods under evaluation. In our case the sensitivity and specificity of exhaled NO and PC_{20}, etc., would be lowered. Here, the imperfect standard bias is of course not known because a true gold standard is lacking. We however compared several test methods within the same database. With the same imperfect standard bias for all, the correction factor will be exactly the same for all tests and the resulting ranking is therefore valid.

Extrapolation and interpretation

The extrapolation of diagnostic studies can be influenced by several problems. We first of all tried to avoid selection bias by randomly inviting new referred subjects before a diagnosis was made: the effect of season, of treating physicians, etc. is minimized in that way. Next to this the sample size (case/variable ratio >20:1) of the study and the a priori selection of modifiers also safeguards against biased outcomes. We therefore feel that our findings can be extrapolated, also because in two centres the findings and comparisons turned out to be very similar.

Still we must emphasize that cut-off values are system-dependent. Because of the fact that eNO levels in the Spaarne hospital were always slightly higher compared to

the UMCU-levels based on systemic instrument differences, the discriminatory power of eNO in the two hospitals is similar but cut-off values will show the same systemic difference. Therefore, we did not supply formulas for correction of the eNO levels.

Our results are in good accordance with the recent findings of Dressel et al.,[24] who did find an influence on eNO of smoking, respiratory tract infection, height, gender and atopy in a large cohort of subjects (steroid users were excluded). The authors advised to correct eNO for these factors.

Several studies found a stronger correlation between atopy (also ''extrapulmonary'') and eNO levels, than asthma and eNO levels,[9,25] although eNO levels were not ln-transformed in these studies. On the other hand, Dressel et al.[24] found no interaction between respiratory allergy, sex, gender, smoking and recent respiratory tract infection in log transformed eNO levels in steroid naïve subjects, and created a correction formula for eNO based on these parameters. In the introduction we quoted some studies in favour of using eNO to guide asthma treatment, recently some papers were published that showed no benefit of using eNO in the clinical setting for guiding therapy.[4,26] In these two studies eNO levels were not corrected for nuisance factors. It would be interesting to re-assess this study with eNO levels corrected for nuisance factors.

Berkman et al.[12] concluded that eNO was 'as good as bronchial provocation tests' in diagnosing asthma, in a very well defined population excluding those with ICS use, obstructive pattern, and significant reversibility. Furthermore, the subjects included were already considered to be possible asthmatics by the referring physician.[12] The same selection bias due to exclusion of subjects on ICS and the inclusion of only subjects highly suspective of being asthmatics was present in the study of Dupont.[13] These bias leads to difficulty in implementation of the results of these two studies in clinical practice.

Rationale of selected methods

Based on the comparison as depicted in Tables 4 and 5 one can assess the relative merits of the methods. The FEV_1-reversibility, the PC_{20} and the corrected eNO-method seem to be equivalent in their capability to diagnose asthma, as their areas under the ROC curve are very comparable. Choosing any of the three methods will deliver the same diagnostic quality. So secondary arguments can be added to the equation, when taking a decision which method to prefer. The advantage of the eNO-method is the quick and non-invasive character, but one needs to correct for modifiers. That isn't needed for the FEV_1-reversibility and, to a lesser extent, for the PC_{20}. Of the three methods the PC_{20} is the one which is most cumbersome to the patient and requires most time: one could therefore view upon this method as least suitable for daily practice use. Based on the minor effect of modifiers on the FEV_1-reversibility, this method is the least complicated.

In conclusion

The measurement of the eNO level is a non-invasive, safe and quick diagnostic for asthma, even in subjects using inhaled steroids, under the condition that a correction for allergy, use of inhaled steroids, recent infection, smoking, sex and the use of nitrate food has been made. In unselected subjects, it is capable of separating asthma from non-asthma comparable to the FEV_1-reversibility.

Conflict of interest statement

The authors have no conflicts of interest to report.

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